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Expression of pituitary tumour transforming gene (PTTG) and fibroblast growth factor-2 (FGF-2) in human pituitary adenomas: relationships to clinical tumour behaviour

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Summary

OBJECTIVE Pituitary tumour transforming gene (PTTG) encodes a multifunctional protein that is implicated in initiating and perpetuating pituitary adenoma growth. PTTG appears to have key regulatory functions in determining control of many fundamental cellular events including mitosis, cell transformation, DNA repair and gene regulation. Several of these events are mediated through interactions with PTTG binding factor (PBF) and fibroblast growth factor-2 (FGF-2). Given this background, we have determined the expression of PTTG, PBF, FGF-2 and its receptor FGF-R-1 in a large cohort of pituitary adenomas and have sought associations between levels of gene expression and clinical markers of tumour behaviour.

PATIENTS AND METHODS We used real-time reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blot analyses to measure PTTG, PBF, FGF-2 and FGF-R-1 expression in *ex vivo* pituitary tumours ($N = 121$). Clinical data, including accurate radiological assessment of tumour characteristics, were used to determine any associations between gene expression and tumour behaviour.

RESULTS PTTG was increased significantly (fivefold, $P = 0{\cdot}005$) in adenomas compared with normal pituitaries. We also demonstrated that PBF was similarly raised in adenomas (sixfold, $P = 0{\cdot}0001$), and was significantly correlated with PTTG expression. FGF-2 and its receptor FGF-R-1 were also raised in adenomas compared with normal pituitary tissue. Moreover, significantly enhanced expression of FGF-R-1 was observed in invasive adenomas compared with other pituitary tumours.

CONCLUSIONS Our data support a fundamental role for PTTG-mediated upregulation of FGF-2 signalling in pituitary tumorigenesis and growth, and suggest that receptor-mediated mechanisms of growth factor action may be critically important. Further prospective studies are required to determine whether measurement of FGF-R-1 mRNA will be of clinical use as a prognostic marker in patients with pituitary adenomas.

Despite intensive study, the mechanisms of sporadic pituitary tumorigenesis are unclear. To date, none of the observations from cellular and molecular studies has directly impacted upon the clinical management of patients with pituitary tumours. In particular, the lack of reliable prognostic indicators for patients with pituitary adenomas makes tumour-specific therapy an unobtainable ideal. Conventional markers of aggressive tumour behaviour, such as measurements of Ki67, bromodeoxyuridine, and proliferating cell nuclear antigen (PCNA) expression (Knosp *et al.*, 1989; Buchfelder *et al.*, 1996; Thapar *et al.*, 1996a,b; Atkin *et al.*, 1997), have all proved disappointing in pituitary adenomas, probably due to the benign nature of these tumours. Furthermore, search for rearrangements or mutations of candidate oncogenes (*myc*, *ras*, *bcl*, *Hst1*, *sea* and *fos*) and tumour suppressor genes (*menin*, *RB1*, *p16*) has also been largely unfruitful in pituitary adenomas. However, a recent report has described a truncated form of fibroblast growth factor (FGF) receptor-4 (ptd-FGFR4) in pituitary adenomas (Ezzat *et al.*, 2002). ptd-FGFR4 exhibited tumorigenic properties *in vivo*, and, with further characterization, this novel oncogene may prove to be an important factor in the complex process of pituitary tumorigenesis.

Characterization of the human pituitary tumour transforming gene (PTTG) (Zhang *et al.*, 1999a) has provided significant insight into its potential roles in pituitary tumorigenesis and also offers a potentially novel prognostic marker for pituitary and other human neoplasms. Originally isolated from rat prolactin-secreting cells (Pei & Melmed, 1997), PTTG is increasingly recognized as

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a multifunctional protein, with roles in the control of mitosis (Zou *et al.*, 1999; Yu *et al.*, 2000; Zur & Brandeis, 2001), cell transformation (Zhang *et al.*, 1999a), DNA repair (Romero *et al.*, 2001) and gene regulation (Zhang *et al.*, 1999a; Pei, 2001). Critical to the functioning of PTTG is its interaction with a recently characterized binding factor (PBF), which serves to translocate PTTG from the cytoplasm to the nucleus (Chien & Pei, 2000). One of the key functions of PTTG in the nucleus is mitotic regulation through its action as a securin homologue (Zou *et al.*, 1999). PTTG must be proteolysed during cell division for sister chromatid separation to occur, and failure of this elicits inappropriate sister chromatid exchange, resulting in genetic instability as an early tumorigenic event (Zou *et al.*, 1999; Yu *et al.*, 2000; Zur & Brandeis, 2001). Indeed, recent studies have identified unusually frequent rates of aneuploidy in high PTTG-expressing MG-63 (Yu *et al.*, 2000) and NIH-3T3 (Zur & Brandeis, 2001) cells. Furthermore, several studies have identified high rates of aneuploidy in pituitary adenomas (Daniely *et al.*, 1998; Hui *et al.*, 1999; Larsen *et al.*, 1999).

Of particular importance to pituitary disease is the observation that PTTG upregulates FGF-2 expression (Zhang *et al.*, 1999a). FGF-2 has previously been implicated in the growth and progression of pituitary adenomas. *In vivo*, FGF-2 regulates GH, PRL and TSH secretion (Baird *et al.*, 1985; Larson *et al.*, 1990), and *in vitro*, pituitary adenomas respond to treatment with human rFGF-2 by increased hormone secretion (Atkin *et al.*, 1993, 1995). Furthermore, abnormal expression of FGF-2 and FGF-receptors (FGF-Rs) has been reported in such tumours (Li *et al.*, 1992; Abbass *et al.*, 1997). A functional link between PTTG, FGF-2 and angiogenesis has also recently been reported (Ishikawa *et al.*, 2001), providing an association between tumour initiation and neovascularization, in turn allowing tumour growth beyond a few millimetres (Folkman, 1990). Taken together, these findings support a role for PTTG-mediated regulation of FGF-2 being fundamental to pituitary tumorigenesis and tumour progression.

Given the substantial and growing evidence that PTTG has multiple key points of action in pituitary tumorigenesis as an early cause of genetic instability through aberrant cell division, and as a promoting factor encouraging tumour growth through FGF-2 induction, there is a rationale for considering its use as a marker of tumour biological behaviour in pituitary adenomas. There is a precedent for this as the previous work by Zhang *et al.* (1999a) described increased PTTG mRNA expression in more invasive functioning (but not nonfunctioning) pituitary adenomas compared with lower grade tumours. Furthermore, we have previously demonstrated significantly increased PTTG mRNA expression in colon cancer with lymph node invasion compared with carcinoma confined to the bowel wall (Heaney *et al.*, 2000).

Given their likely importance in pituitary tumorigenesis and their potential applicability as markers of tumour behaviour and thus prognosis, we have undertaken a detailed assessment of the expression of PTTG, PBF, FGF-2 and its receptor FGF-R-1 (the most frequently expressed FGF-R in human pituitary; Abbass *et al.*, 1997) in a large cohort of normal and tumorous human pituitaries. Furthermore, we have related our *ex vivo* gene expression findings to clinical parameters of pituitary tumour behaviour, to determine whether such genes may be useful in predicting further pituitary tumour growth.

Materials and methods

Patients and pituitary tissues

With approval of the local research ethics committee, samples of pituitary tumour tissue from 121 patients with pituitary adenomas were obtained at the time of surgery and immediately snap frozen. Clinical details of patients are summarized in Table 1. All tumours were fully characterized by immunohistochemical staining for anterior pituitary hormones. Ninety-two (77%) were clinically nonfunctioning tumours (NFTs) and all of these were macroadenomas (≥ 1 cm in diameter). NFTs stained variably for the glycoprotein hormone common α-subunit and other glycoprotein hormone-specific β-subunits. Functioning pituitary tumours comprised 16 GH-secreting (12 macroadenomas), five PRL-secreting (three macroadenomas), five ACTH-secreting (one macroadenoma) and three TSH-omas (all macroadenomas). Ten normal human pituitary glands were obtained from post-mortem proceedings performed within 24 h of the time of death.

RNA extraction and reverse transcription

Total RNA was extracted from about 100 mg of tissue after homogenization, using the Sigma Trisol kit – a single-step acid guanidinium phenol–chloroform extraction procedure – following the manufacturer's guidelines. RNA was reverse transcribed using Avian Myeloblastosis virus (AMV) reverse transcriptase

Table 1 Details of complete cohort of patients ($N = 121$) showing tumour subtype, mean age (± SE) at diagnosis, number (%) of females, and number (%) of macroadenomas (≥ 1 cm diameter). Following determination of potential tumour contamination with normal pituitary tissue (see Patients and Methods), we excluded one GH-, one PRL- and one ACTH-secreting tumour, and seven NFTs

	Age ± SE (years)	Females (%)	Macroadenomas
All tumours ($N = 121$)	58·9 ± 1·3	62 (51)	114 (94)
Nonfunctioning ($N = 92$)	61·4 ± 1·4	43 (47)	92 (100)
GH-secreting ($N = 16$)	55·0 ± 3·0	11 (69)	12 (75)
PRL-secreting ($N = 5$)	37·7 ± 0·3	3 (60)	3 (60)
ACTH-secreting ($N = 5$)	45·5 ± 6·1	3 (60)	1 (20)
TSH-secreting ($N = 3$)	56·0 ± 6·1	1 (33)	3 (100)

Table 2 Oligonucleotide sequences of PCR primers and TaqMan™ probes used. All TaqMan primers run at 59°C and yield amplicons of 70–150 bp. Pit1 annealed at 60°C, and POMC at 62°C

	Forward primer	Reverse primer
PTTG	GAGAGAGCTTGAAAAGCTGTTTCAG Probe: TGGGAATCCAATCTGTTGCAGTCTCCTTC	TCCAGGGTCGACAGAATGCT
PBF	CTCTTCTCAGTTTGTGAAACGCTAA Probe: AAGCCGTGACGGCACCCAGC	CTGCCCTGGGAGAATGACA
FGF-2	CGACCCTCACATCAAGCTACAA Probe: CGACCCTCACATCAAGCTACAA	CCAGGTAACGGTTAGCACACACT
FGF-R-1	GTGGTGTTGGCAGAGGCTATC Probe: CTGGACAAGGACAAACCCAACCGTG	TCTGTTGCGTCCGACTTCAA
Pit1	AGCAGCGGTTCTCCTTATTTTT	CTTTTCCGCCTGAGTTCCTG
POMC	GGGCAAGCGCTCCTACTCCAT	TGCCCTCACTCGCCCTTCTTGT

(Promega, Madison, WI, USA) in a total reaction volume of 20 µl, with 1 µg of pituitary total RNA, 30 pmol oligo(dT)15, 4 µl of 5 × AMV reverse transcriptase buffer, 2 µl of deoxynucleotide triphosphate (dNTP) mix (200 mM each), 20 units of ribonuclease inhibitor (Rnasin®, Promega) and 15 units of AMV reverse transcriptase (Promega).

Contamination with normal pituitary tissue

To determine whether there was significant contamination of NFTs and ACTH-secreting adenoma samples with normal pituitary tissue, we assessed mRNA expression for Pit-1 as described previously (Alexander *et al.*, 1996; Gittoes *et al.*, 1997). Seven NFTs and one ACTH-secreting tumour showed evidence of significant Pit-1 expression, and were thus excluded from further analyses. To examine normal tissue contamination of other types of functioning tumour, we assessed proopiomelanocortin (POMC) mRNA expression, which is not present in tumours of the somatotroph/lactotroph lineages. These experiments led us to eliminate one GH-secreting and one PRL-secreting tumour from our cohort. In total, therefore, we excluded 10 of our 121 tumours, giving a final cohort size of 111 samples.

Quantitative polymerase chain reaction (PCR)

Expression of specific messenger RNAs was determined using the ABI PRISM 7700 Sequence Detection System. Reverse transcriptase-polymerase chain reaction (RT-PCR) was carried out in 25-µl volumes on 96-well plates, in a reaction buffer containing 1 × TaqMan Universal PCR Master Mix, 3 mM $Mn(OAc)_2$, 200 µM dNTPs, 1·25 U AmpliTaq Gold polymerase, 1·25 U AmpErase UNG, 100–200 nmol TaqMan probe and 900 nmol primers. All reactions were multiplexed with a preoptimized control probe for 18S ribosomal RNA (PE Biosystems, Warrington, UK), enabling data to be expressed in relation to an internal reference, to allow for differences in RT efficiency. Primer and probe sequences are given in Table 2. According to the manufacturer's guidelines, data were expressed as Ct values (the cycle number at which logarithmic PCR plots cross a calculated threshold line) and used to determine ΔCt values (ΔCt = Ct of the target gene (e.g. PTTG) minus Ct of the housekeeping gene). To exclude potential bias due to averaging data that had been transformed through the equation $2^{-\Delta\Delta Ct}$ to give fold changes in gene expression, all statistics were performed with ΔCt values. Measurements were carried out a minimum of three times each. Samples that failed to show consistent, repeatable gene expression were excluded from subsequent analyses. All target gene probes were labelled with FAM, and the housekeeping gene with VIC. Reactions were as follows: 50°C for 2 min, 95°C for 10 min; then 44 cycles of 95°C for 15 s and 60°C for 1 min.

Western blots

Proteins were prepared from 24 pituitary specimens (chosen on the basis of substantial amounts of tissue being available) in lysis buffer (100 mmol/l sodium chloride, 0·1% Triton X-100, and 50 mmol/l Tris, pH 8·3) containing enzyme inhibitors (1 mmol/l phenylmethylsulphonylfluoride, 0·3 µmol/l aprotinin and 0·4 mmol/l leupeptin) and denatured (2 min, 100°C) in loading buffer. Protein concentration was measured by the Bradford assay with bovine serum albumin as standard. Western blot analyses were performed as described previously (Gittoes *et al.*, 1997; Heaney *et al.*, 2000). Briefly, soluble proteins (30 µg) were separated by electrophoresis in 12·5% sodium dodecyl sulphate polyacrylamide gels, transferred to polyvinylidene fluoride membranes, incubated in 5% nonfat milk in phosphate-buffered saline with 0·1% Tween, followed by incubation with antibodies to FGF-2 or FGF-R-1 (both at 1 : 1000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 16 h at 4°C. A PTTG antibody (1 : 5000) was used as described previously (Heaney *et al.*, 2000). After washing in phosphate-buffered saline plus 0·1%

Tumour	Mean age ± SE (years)	Sex (female)	Recurrent	Hypopituitary	Oestrogen deficiency
All (87)	59·4 ± 1·5	45 (52%)	9 (10%)	14 (16%)	12 (27%)
NFT (66)	61·1 ± 1·6	33 (50)	8 (12)	9 (14)	8 (24)
GH (14)	56·9 ± 3·4	9 (64)	1 (7)	5 (36)	2 (22)
PRL (4)	37·5 ± 0·5	2 (50)	0 (0)	0 (0)	2 (100)
ACTH (2)	47·5 ± 2·5	1 (50)	0 (0)	0 (0)	0 (0)
TSH (1)	55	0 (0)	0 (0)	0 (0)	N/A

Table 3 Patient details and evidence of tumour regrowth (recurrent), hypopituitarism preoperatively (hypopituitary) and oestrogen deficiency preoperatively (in females) in patients in whom radiological assessment of tumour size and invasion was made. Recurrent refers to tumour regrowth that necessitated further surgery and/or pituitary radiotherapy in the period following molecular examination of adenoma tissue

Tween, blots were incubated with appropriate secondary antibodies conjugated to horseradish peroxidase for 1 h at room temperature. After further washes, antigen–antibody complexes were visualized by the ECL chemiluminescence detection system (Amersham, Buckinghamshire, U.K.). Actin expression was determined in normal and tumour samples, and revealed no obvious differences in protein loading between the two groups (see Results). Coomassie Blue staining demonstrated a similar finding (data not shown). There are currently no available PBF antibodies, and hence this gene could not be assessed at the protein level.

Association between gene expression and clinical findings

Detailed analyses of preoperative magnetic resonance imaging (MRI) scans were made in 87 (74%) patients in whom informative data had been obtained from molecular studies on pituitary adenoma tissue. The baseline clinical characteristics of patients in this subgroup were not significantly different from the total cohort (see Table 3 for summary of clinical data). Pituitary tumour volume was calculated in coronal and sagittal planes from MRI scans performed at initial clinical presentation. The previously validated SIPPAP classification (Edal *et al.*, 1997) was used to score pituitary tumours for evidence of extension and invasion into surrounding structures. Tumours were also graded using the modified Hardy–Wilson criteria (Wilson, 1990). Patient's demographic details and pituitary function pre- and postoperatively were recorded, as was a history of previous surgery or radiotherapy for a pituitary tumour. Data were examined to determine any associations between clinical tumour behaviour and pituitary gene expression.

Statistical analyses

Data were analysed using Minitab version 13 software. Student's *t*-test and the Mann–Whitney *U*-test were used for comparison between two groups of parametric and nonparametric data, respectively. The Kruskal–Wallis test was used for between-group comparison of more than two groups of nonparametric data. Correlations between levels of mRNA expression were performed using the Pearson Rank Sum test. The χ^2-test was used to analyse prevalence data. Significance was taken as $P < 0{\cdot}05$.

Results

PTTG and PBF expression

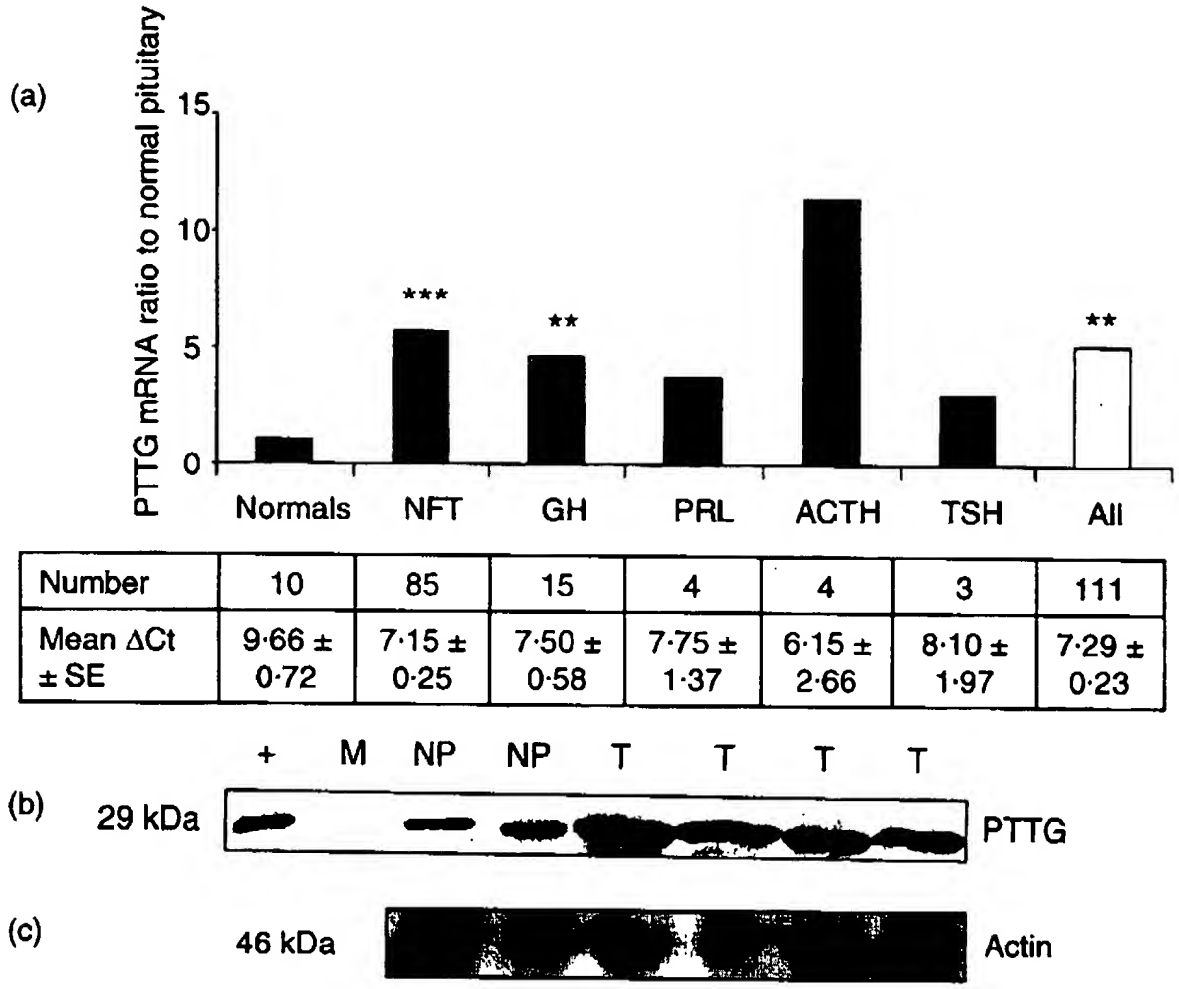
PTTG mRNA expression was increased (about fivefold) in pituitary tumours as a whole ($N = 111$), compared with control normal pituitary tissue ($N = 10$, $P = 0{\cdot}005$, Fig. 1a). Nonfunctioning tumours and somatotrophinomas showed similar increases in PTTG mRNA (5·7-fold, $P = 0{\cdot}002$; 4·6-fold, $P = 0{\cdot}022$, respectively). Western blot analysis (Fig. 1b) showed that PTTG protein expression was consistent with the mRNA findings of raised expression in tumours compared with normals.

PBF mRNA expression was also markedly increased in pituitary tumours of all subtypes ($n = 98$) compared with normal pituitary tissue (5·7-fold increase, $P < 0{\cdot}0001$, Fig. 2a); NFTs exhibited particularly high PBF expression (6·6-fold compared with normals, $P < 0{\cdot}0001$). A significant positive correlation ($P = 0{\cdot}0001$, $R = 0{\cdot}4$) between the expression of PTTG and that of PBF mRNA expression was observed in pituitary tumours (Fig. 2a), but not in normal pituitaries ($P = 0{\cdot}342$; data not shown).

FGF-2 and FGF-R-1 expression in pituitary tumours

FGF-2 mRNA expression was similar in pituitary tumours and normal pituitaries (relative expression = 0·84, P = N/S, Fig. 3a). Interestingly, TSH-omas showed significantly reduced expression (approximately 90% reduction) of FGF-2 compared with normals ($P = 0{\cdot}017$). Although FGF-2 mRNA was not increased in tumours, Western blot analysis revealed that tumours consistently expressed more FGF-2 protein than normal pituitary tissue (Fig. 3b). However, because of the small size of our TSH-omas, we were unable to determine FGF-2 protein expression in this tumour subtype.

The FGF-2 receptor FGF-R-1, unlike its ligand, demonstrated raised mRNA expression in tumours (Fig. 4a). Pituitary tumours overall showed a 3·6-fold increase ($P < 0{\cdot}05$), with NFTs ($N = 83$) demonstrating a 6·2-fold induction of FGF-R-1 mRNA ($P < 0{\cdot}005$) compared with normals. Consistent with the marked reduction in FGF-2 mRNA apparent in TSH-omas, these tumours ($N = 3$) also expressed very low levels of FGF-R-1, with a mean reduction of 97% compared with normal pituitaries ($P = 0{\cdot}001$).



Number	10	85	15	4	4	3	111
Mean ΔCt ± SE	9·66 ± 0·72	7·15 ± 0·25	7·50 ± 0·58	7·75 ± 1·37	6·15 ± 2·66	8·10 ± 1·97	7·29 ± 0·23

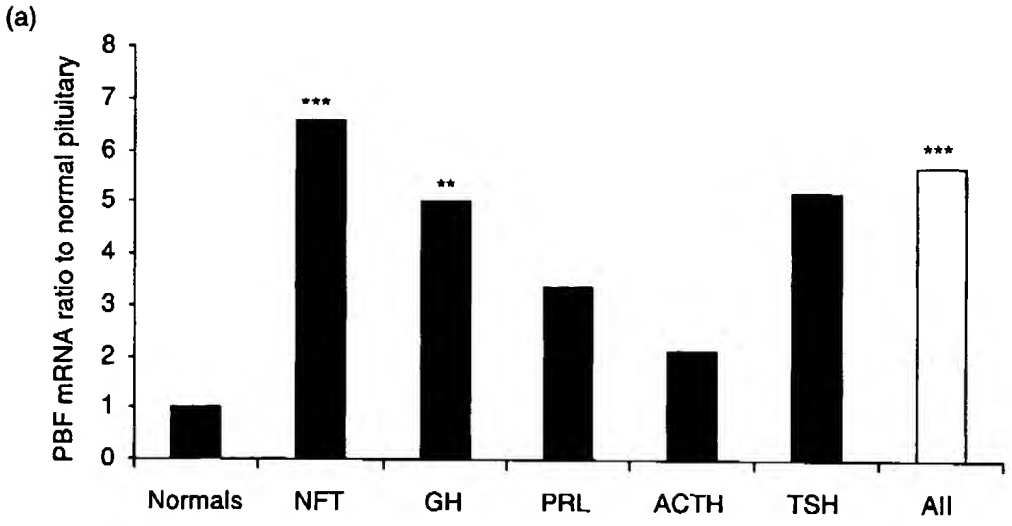
Fig. 1 (a) PTTG mRNA expression in 10 normal and 111 tumorous pituitaries (All). NFT, nonfunctioning tumours ($N = 85$); GH, somatotrophinomas ($N = 15$); PRL, prolactinomas ($N = 4$); ACTH, adrenocorticotrophinomas ($N = 4$); TSH, thyrotrophinomas ($N = 3$). $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$, compared with normals. In this and following histograms, gene expression in tumours is displayed relative to a value of 1·0 for normal pituitaries. Number of samples and ΔCt values (± SEM) are given below each histogram bar. (Note that low ΔCt values represent high gene expression.) (b) Representative Western blot analysis of PTTG protein expression in two normal pituitaries (NP) and four nonfunctioning tumours (T). M, size marker; +, JEG-3 cell extract as a positive control for PTTG expression. (c) Representative actin expression in the pituitary extracts assayed for PTTG, demonstrating consistent protein loading for normal and tumorous samples.

Findings from Western blot analyses of FGF-R-1 protein paralleled the pretranslational increases in expression of this gene in pituitary tumours (Fig. 4b).

Associations between gene expression and clinical findings

Given the marked upregulation of each of the genes studied in pituitary tumours, data were analysed for all pituitary tumours and for pituitary tumour subtypes to determine any associations between clinical parameters and measures of gene expression. Pituitary tumours were classified according to the SIPPAP criteria (see Patients and Methods). No significant associations were observed between PTTG or PBF mRNA expression and clinical variables (age, sex, oestrogen status, degrees of hypopituitarism preoperatively, and presence of recurrent tumour growth requiring further treatment) or radiological parameters of tumour invasion.

When all tumours were analysed together, FGF-R-1 mRNA was expressed at increased levels in higher-grade pituitary tumours (as determined by Zhang *et al.*, 1999a). Grade 1 tumours ($N = 11$, < 1 cm diameter) expressed similar levels of FGF-R-1 mRNA to normal pituitaries, whereas grade 2 tumours ($N = 18$,



Number	10	75	12	4	4	3	98
Mean ΔCt ± SE	11·14 ± 0·52	8·42 ± 0·19	8·84 ± 0·53	9·75 ± 0·35	10·05 ± 0·67	8·76 ± 2·56	8·63 ± 0·17

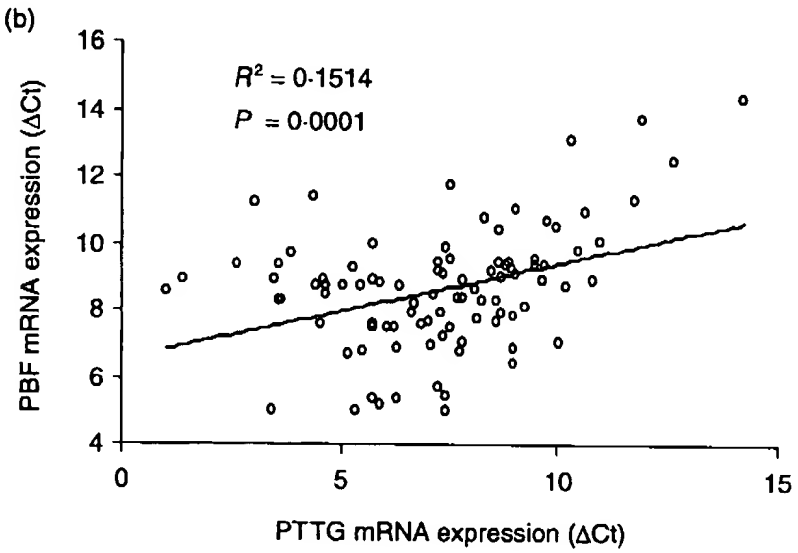
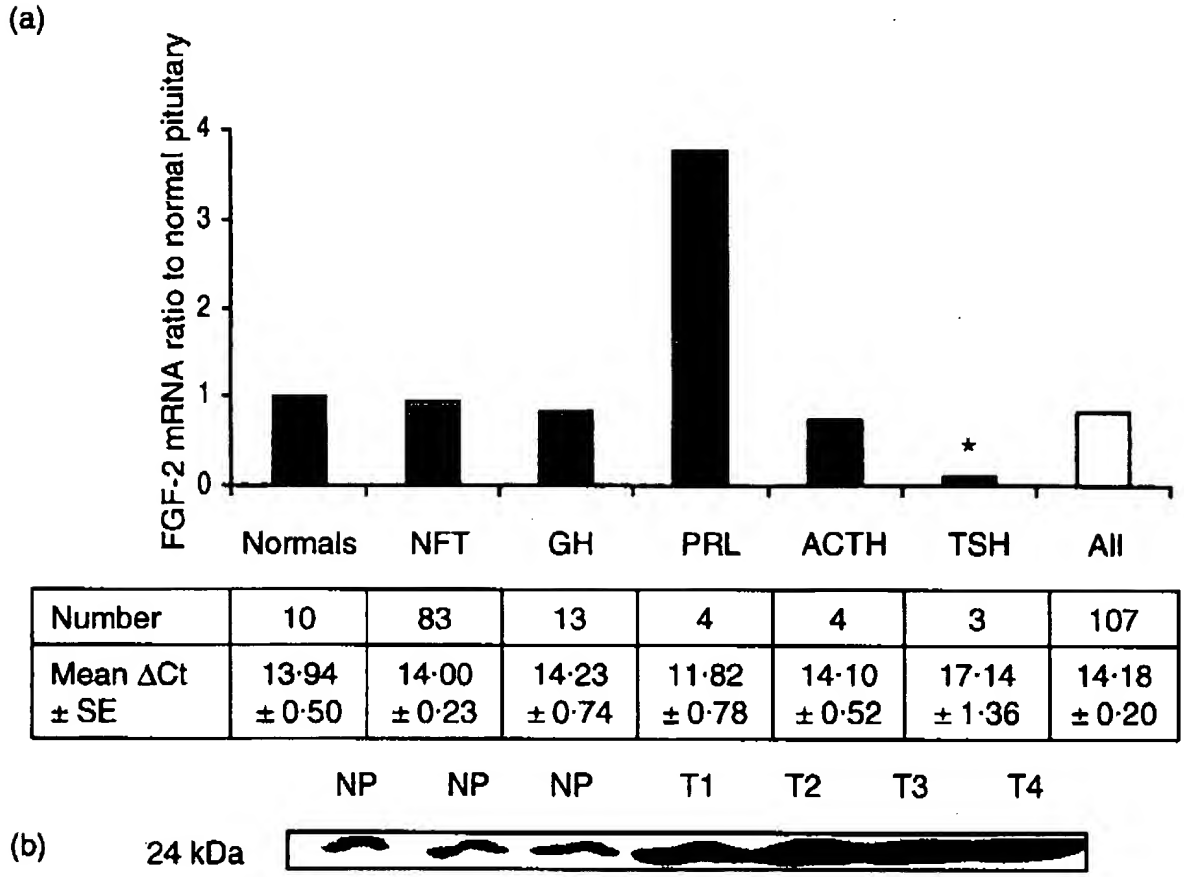


Fig. 2 (a) PBF mRNA expression in normal and tumorous pituitaries. $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$, compared with normal ($N = 10$). (b) Correlation between PTTG and PBF mRNA expression in pituitary tumours of all subtypes analysed together. Data are given as ΔCt values to exclude potential bias due to averaging data transformed through the equation $2^{-\Delta\Delta Ct}$. PTTG and PBF expression demonstrated a highly significant ($P = 0{\cdot}0001$) positive correlation, indicating that tumours with high PTTG expression (i.e. low ΔCt values) also had high levels of PBF mRNA.

≥ 1 cm with sellar enlargement) expressed 4·6-fold (median, range 0·05–83·3), grade 3 tumours ($N = 56$, with evidence of localized perforation of sellar floor) 7·5-fold (median, range 0·01–171·3), and grade 4 tumours ($N = 2$, diffuse destruction of sellar floor) 189-fold (median, range 25–353) greater expression than normal pituitaries ($P < 0{\cdot}05$, Kruskal–Wallis, Fig. 5).

As a group, functioning tumours showed increased FGF-R-1 mRNA expression in tumours with evidence of infrasellar



Number	10	83	13	4	4	3	107
Mean ΔCt ± SE	13·94 ± 0·50	14·00 ± 0·23	14·23 ± 0·74	11·82 ± 0·78	14·10 ± 0·52	17·14 ± 1·36	14·18 ± 0·20

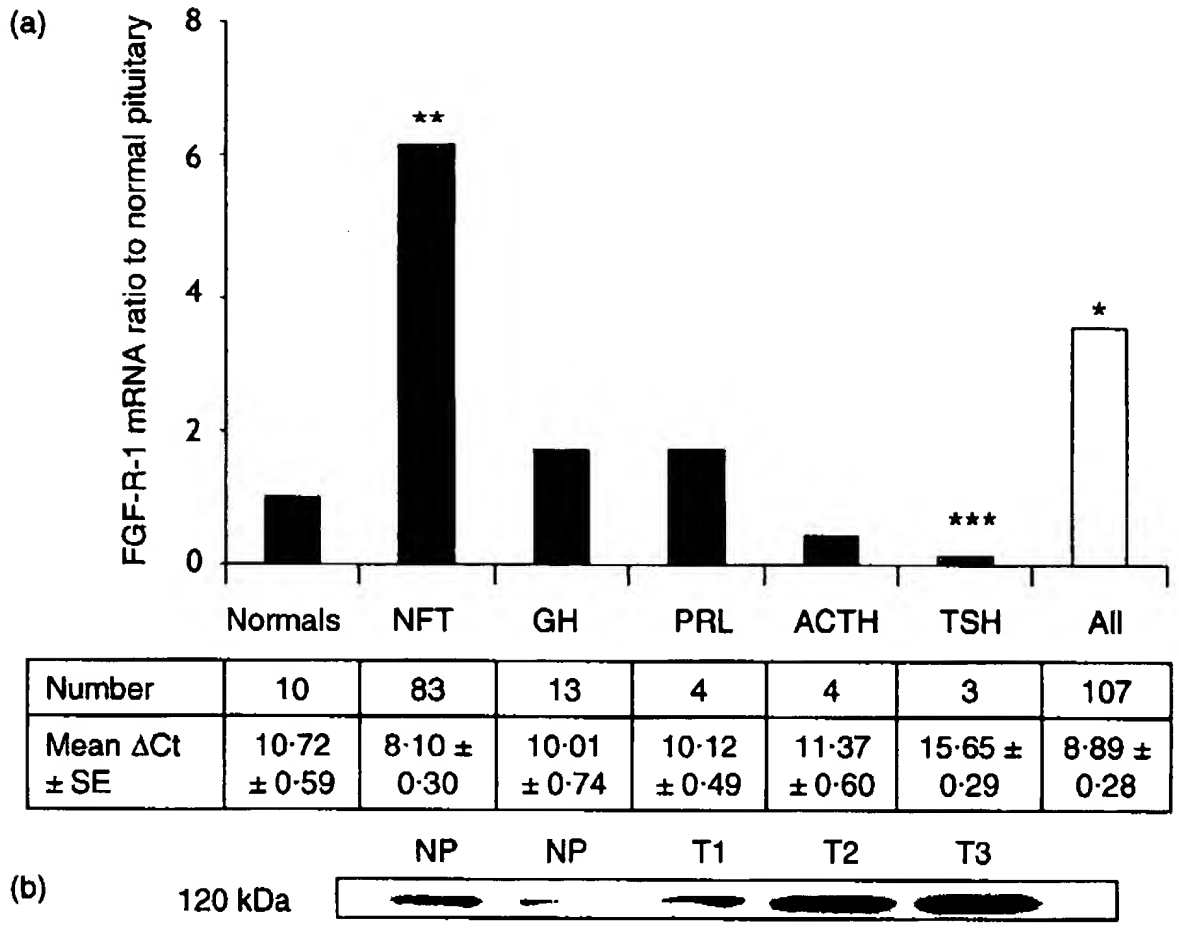
Fig. 3 (a) FGF-2 mRNA expression in normal and tumorous pituitaries. No tumour subtype showed evidence of raised FGF-2 mRNA, although TSH-omas demonstrated significantly lowered expression (*$P = 0{\cdot}017$, $N = 3$). (b) Representative Western blot analysis of FGF-2 protein expression in three normal pituitaries (NP) and four representative NFTs (T), indicating that protein expression of this gene was higher in tumours than normal pituitaries.

extension (median 2·5-fold increase, range 0·20–25·19) compared with those without (median 1·06-fold, range 0·02–4·16, $P < 0{\cdot}05$, Kruskal–Wallis test), suggesting that FGF-R-1 mRNA expression may be a marker of sphenoid bone invasion.

Discussion

PTTG is a multifunctional proto-oncogene that is implicated in several mechanisms of tumour initiation and progression. The consistent finding of PTTG overexpression in our large cohort of pituitary tumours supports a link between increased PTTG expression and pituitary tumorigenesis, although it remains to be determined whether this is directly causative *in vivo*. PTTG signals through interaction with the binding factor PBF, which, following PTTG phosphorylation, mediates its nuclear translocation. For the first time, we demonstrated increased pretranslational expression of PBF in pituitary adenomas. FGF-2 induction by PTTG is also likely to be a central event in perpetuating pituitary adenoma growth and we have shown increased levels of FGF-2 protein in adenomas compared with normal pituitaries. Similarly, we found the FGF-2 receptor FGF-R-1 was highly expressed in tumours compared with normal pituitaries. Furthermore, we noted significantly increased FGF-R-1 mRNA expression in higher grade tumours, and in functioning tumours that invaded the sphenoid bone compared with those that did not, thus raising the possibility of using such molecular markers as indicators of tumour biological behaviour. Because aggressive pituitary tumours are rare, however, further prospective studies to generate larger numbers are required to determine whether an association between tumour grade and FGF-R-1 mRNA expression will have any clinical relevance.

Because of the uniform finding of high expression of PTTG in all cell lines so far examined (Zhang *et al.*, 1999b), PTTG is likely to be an important oncogene (acting via overexpression) in a number of human malignancies. In colorectal carcinomas, we have previously reported higher PTTG expression in tumours



Number	10	83	13	4	4	3	107
Mean ΔCt ± SE	10·72 ± 0·59	8·10 ± 0·30	10·01 ± 0·74	10·12 ± 0·49	11·37 ± 0·60	15·65 ± 0·29	8·89 ± 0·28

Fig. 4 (a) Pretranslational FGF-R-1 expression in normal and tumorous pituitaries. $*P < 0{\cdot}05$, $**P < 0{\cdot}01$, $***P < 0{\cdot}001$. Tumours overall and NFTs as a subgroup ($N = 83$) showed significantly increased mRNA levels of this gene compared with normals. GH-secreting, PRL-secreting and ACTH-secreting tumours did not exhibit increased FGF-R-1 mRNA overexpression. Interestingly, TSH-omas demonstrated a 97% decrease in FGF-R-1 mRNA expression compared with normal pituitaries ($P = 0{\cdot}001$, $N = 3$). (b) Representative Western blot analysis of FGF-R-1 protein in two normal pituitary (NP) and three nonfunctioning tumours (T1–T3), showing upregulation of FGF-R-1 in tumours.

with lymph node invasion than those confined to the bowel wall (Heaney *et al.*, 2000). In the context of benign pituitary adenomas, PTTG mRNA expression has also been related to the degree of invasion into the sphenoid bone in functioning pituitary tumours (Zhang *et al.*, 1999a). In that study, PTTG mRNA expression was determined by semiquantitative RT-PCR in 30 NFTs and 24 functioning pituitary tumours (Zhang *et al.*, 1999a). Invasion was assessed by a variety of parameters including MRI findings, surgical observations and pathological data. In our much larger cohort of pituitary adenomas, gene expression was determined by quantitative RT-PCR. Using information from pituitary MRI scans and the SIPPAP classification (Edal *et al.*, 1997), we found no relationship between PTTG mRNA expression and markers of tumour extension or invasion. Also, although oestrogen induction of PTTG has been described previously (Heaney *et al.*, 1999), we detected no difference in PTTG mRNA expression between males and females or between eugonadal or hypogonadal females. We did, however, note increased FGF-R-1 mRNA expression in functioning tumours that invaded the sphenoid bone compared with those that did not. This is pertinent given the well-documented association between PTTG and FGF-2 regulation, and suggests that increased FGF-2 signalling through enhanced FGF-R-1 expression may be responsible for the more invasive phenotype of these tumours. As FGF-2 does not affect expression of FGF-R-1 mRNA (Grothe *et al.*, 1997), it is possible that PTTG directly or indirectly regulates FGF-R-1 expression to mediate enhanced FGF-2 effects.

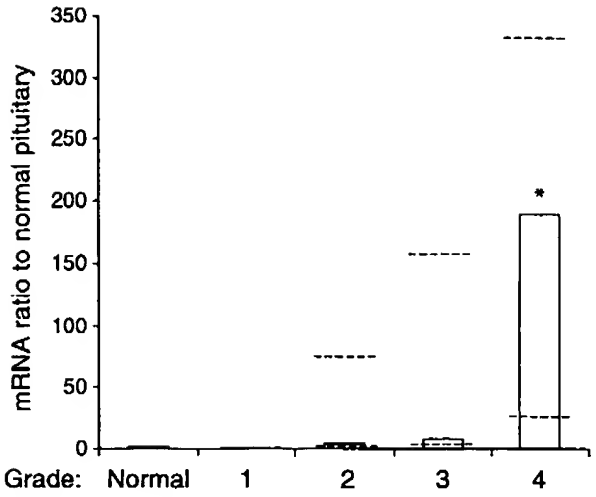


Fig. 5 FGF-R-1 mRNA expression in pituitary tumours. FGF-R-1 mRNA was expressed at increased levels in higher grade pituitary tumours [as determined by Zhang *et al.* (1999a)]. Grade 1 tumours (< 1 cm diameter) expressed similar levels of FGF-R-1 mRNA to normal pituitaries, whereas grade 2 tumours (≥ 1 cm with sellar enlargement) expressed 4·6-fold [median (histogram bars), range 0·05–83·3 (dashed lines)], grade 3 tumours (with evidence of localized perforation of sellar floor) 7·5-fold (median, range 0·01–171·3), and grade 4 tumours ($N = 2$, diffuse destruction of sellar floor) 189-fold (median, range 25–353) greater expression than normal pituitaries (*$P = 0·05$, Kruskal–Wallis).

FGF-2, which is a potent mitogenic and angiogenic factor in a number of human malignancies, may also directly regulate pituitary cell growth, as well as the transcription and secretion of PRL, GH and TSH (Baird *et al.*, 1985; Larson *et al.*, 1990). Recently, a seemingly direct link between PTTG, FGF-2 and angiogenesis has been reported and an association between tumour vascularity and increased PTTG expression was recognized (Ishikawa *et al.*, 2001). Further studies showed that PTTG-stimulated angiogenesis *in vitro* could be abrogated by an anti-FGF-2 antibody. Although we found no increase in FGF-2 mRNA expression in our tumours, we did find greater expression of FGF-2 protein and we propose that this discrepancy reflects differential post-transcriptional regulation of FGF-2. TSH-omas demonstrated particularly interesting FGF-2 signalling, having significantly reduced FGF-2 and FGF-R-1 mRNA expression compared with normal pituitaries, suggesting that TSH-omas may represent a distinct pathogenic pathway of pituitary tumour formation. Alternatively, these observations may simply reflect pituitary cell type specific differences in FGF-2 and FGF-R-1 expression in clonally expanded thyrotrophs.

PBF expression has not previously been described in pituitary tumours. For the first time, we have demonstrated that PTTG mRNA expression is positively associated with that of its binding factor, and that PBF is significantly raised in tumours. These data also indicate the likely existence of a regulatory mechanism coordinating expression of the oncogene and its binding factor. Overall, our data support a fundamental role for PTTG-mediated upregulation of FGF-2 signalling in pituitary tumorigenesis and growth, and suggest that receptor-mediated mechanisms of growth factor action may be critically important. Further elucidation of the mechanisms underlying these described changes in gene expression will be fundamental to determining the poorly understood phenomenon of pituitary tumorigenesis, and may eventually facilitate a gene therapy approach in patients at high risk of tumour recurrence after initial surgical debulking (Castro *et al.*, 1999). Further work involving larger numbers and a prospective analysis need to be performed to determine whether measurement of FGF-R-1 mRNA will be of use as a prognostic marker in patients with functioning pituitary adenomas.

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